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Evidence for distinct chronic wasting disease (CWD) strains in experimental CWD in ferrets

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Chronic wasting disease (CWD) is an evolving prion disease of cervids (deer, elk and moose) that has been recognized in North America and Korea. Infection of non-cervid reservoir or transport species in nature is not reported. However, the ferret (*Mustela putorius furo*) is susceptible to CWD after experimental inoculation. Here, we report that infection of ferrets with either of two ferret CWD isolates by various routes of exposure has revealed biologically distinct strain-like properties distinguished by different clinical progression and survival period. The isolates of ferret CWD were also differentiated by the distribution of the infectious prion protein (PrP^{CWD}) in the brain and periphery, and by the proteinase K sensitivity of PrP^{CWD}. These findings suggest that diversity in prion conformers exists in CWD-infected cervids.

INTRODUCTION

Chronic wasting disease (CWD) of deer and elk is a transmissible spongiform encephalopathy (TSE) of North American cervids, including mule deer, white-tailed deer, elk and moose (Baeten *et al.*, 2007; Sigurdson, 2008; Williams, 2005; Williams & Young, 1980). CWD has been detected in 14 US states and two Canadian provinces, not all of which are contiguous (http://www.nwhc.usgs.gov/disease_information/chronic_wasting_disease/index.jsp) and has been detected in Korea (Kim *et al.*, 2005; Sohn *et al.*, 2002). While the origin of CWD is uncertain, the endemic focus of CWD is in Northern Colorado/Southern Wyoming. Spread has followed natural migration of deer and been extended due to human intervention and trade. CWD is spread horizontally with efficiency (Williams & Miller, 2002) both by direct contact and environmental contamination (Mathiason *et al.*, 2006, 2009; Safar *et al.*, 2008). This facile transmission of the causative prion has raised questions regarding its potential to transgress species barriers. The studies conducted in larger species likely to encounter CWD-infected cervids in nature indicate varying susceptibility of mustelids, raccoons, cats, domestic ruminants and non-human primates following intra-cerebral (IC) challenge (Bartz *et al.*, 1998; Hamir *et al.*, 2001, 2003, 2005, 2006a, 2007; Marsh *et al.*, 2005). The search for smaller species that may act as reservoir or accidental hosts has been more recently undertaken using direct (Heisey *et al.*, 2010) and indirect methods (Kurt *et al.*, 2009). Both studies support a potential role for native rodents in the infection cycle.

While native species may, in the future, provide diverse models for CWD research, well-characterized species are sought for initial studies. The host range of CWD varies from that of scrapie, based on the early work of Bruce *et al.* (2000), which indicated that, unlike scrapie, laboratory mouse strains and hamsters were minimally if at all susceptible to CWD. Bartz and colleagues demonstrated that ferrets could be infected following IC inoculation and that ferret-adapted CWD could be transmitted to hamsters (Bartz *et al.*, 1998). While both ferrets and hamsters are well adapted to laboratory conditions, the more direct susceptibility of ferrets was a distinct advantage in our investigation into the host range and potential reservoirs of CWD. We describe here further work on transmission of CWD in ferrets and evidence for strain-like properties of CWD isolates when adapted to ferrets.

Supplementary material is available with the online version of this paper.

RESULTS

Transmission results in the University of Wisconsin (UWI) isolate by different routes and subpassages

IC inoculation. Each ferret (n=2) inoculated with freezer-archived, ferret CWD from the UWI developed clinical CWD during the 9 months (range=9.6–9.7) after IC

inoculation (Table 1). The clinical course of infection was ⩾3 weeks. In subsequent passages infected ferrets (n=9) showed a 100 % attack rate (AR) and very similar survival (range=8.4–11.7 months). There was no significant difference in survival period between the passage groups (P=0.072, P=0.0584; parametric and non-parametric statistics, respectively) (Fig. 1a). The UWI isolate appeared stably adapted.

Peripheral inoculation. Intra-peritoneal (IP) inoculation of ferrets (n=5) with the UWI isolate resulted in a 100 % AR but variable clinical progression and survival periods (range=9.0–15.1 months). Inoculation of ferrets (n=4) *per os* (PO) resulted in a 100 % AR, a longer incubation period and tighter grouping of survival (range=15.0–15.9 months) (Fig. 1c). The longer incubation period for PO exposure was expected.

Transmission results in the Colorado State University (CSU) isolate by different routes and subpassages

IC inoculation. There was a 100 % AR by this route. Primary passage of CWD into ferrets (n=3) produced a variable survival period (range=14.8–20.25 months) (Table 1, Fig. 1b). Passage two ferrets (n=3) showed a substantially reduced survival period (range=4.6–4.8 months). Passage three ferrets (n=4) showed a further reduction (range=3.5–3.7 months). The total survival range was 3.5–20.25 months and each passage differed significantly (P<0.0001, P=0.0181; parametric and non-parametric statistics, respectively).

Peripheral inoculation. In passage three, inoculation of ferrets (n=4) by the IP route produced a 100 % AR and a short incubation period (range=4.2–4.7 months) as did inoculation of ferrets (n=4) by the PO route (range=6.0–6.6 months). However, one of the four ferrets survived oral challenge resulting in an AR of 75 % for this route of exposure (Fig. 1d).

Clinical presentation of CWD in ferrets inoculated with the UWI isolate

Early signs of CWD were decreased arousal, alertness and exploratory behaviour, followed by reduced food consumption and grooming. Subsequently, motor dysfunction was seen as hindquarter or lower spinal ataxia with a wide-based stance. Neurological signs progressed to generalized ataxia including crossing of front legs, swaying of the neck, head bobbing and lowered head carriage. Less consistent signs included pruritus, aggressiveness and hyperphagia.

Clinical presentation of CWD in ferrets inoculated with the CSU isolate

The presentation of clinical disease in ferrets inoculated with the original mule deer brain pool has been described previously (Bartz *et al.*, 1998; Sigurdson *et al.*, 2008). Clinical signs in ferrets infected with the first passage of the CSU isolate overlapped with those observations given above for the UWI isolate. On second passage, there was a reduced range of clinical presentations. Somnolence and decreased alertness characterized the onset of disease. Affected ferrets appeared depressed and difficult to arouse. The most notable neurological sign was a pronounced intention tremor and ataxia. Ataxia developed rapidly in all ferrets at the second and third passage. There was no observable difference in the character of the clinical signs of CWD in ferrets inoculated by a peripheral route. Ferrets rapidly became terminally affected and moribund.

Clinical course and progression of CWD in ferrets

The difference between the isolates was readily apparent in terms of the progression of disease. Ferrets infected

Table 1. Passage history of two different isolates of ferret CWD

UWI, University of Wisconsin isolate of CWD; CSU, Colorado State University isolate of CWD.

Strain/passage	Inoculation route*	Attack rate†	Survival period in months (mean)	Clinical course
UWI passage three	IC	2/2 (100 %)	9.6–9.7 (9.65)	Steadily progressive
UWI passage four	IC	5/5 (100 %)	9.5–11.7 (10.1)	Steadily progressive
UWI passage four	IP	5/5 (100 %)	9.0–15.1 (11.9)	Variable/progressive
UWI passage four	PO	3/3 (100 %)	15.0–15.9 (15.6)	Steadily progressive
UWI passage five	IC	4/4 (100 %)	8.4–9.1 (8.7)	Steadily progressive
CSU passage one	IC	3/3 (100 %)	14.8–20.25 (18.0)	Variable/progressive
CSU passage two	IC	3/3 (100 %)	4.6–4.8 (4.66)	Short/quick
CSU passage three	IC	4/4 (100 %)	3.5–3.7 (3.61)	Short/quick
CSU passage three	IP	4/4 (100 %)	4.2–4.7 (4.49)	Short/quick
CSU passage three	PO	3/4 (75 %)	6.0–6.6 (6.33)	Short/quick

*IC, Intra-cerebral; IP, intra-peritoneal; PO, *per os*.

†Attack rate expressed as: number of ferrets succumbing to CWD/number of ferrets inoculated.

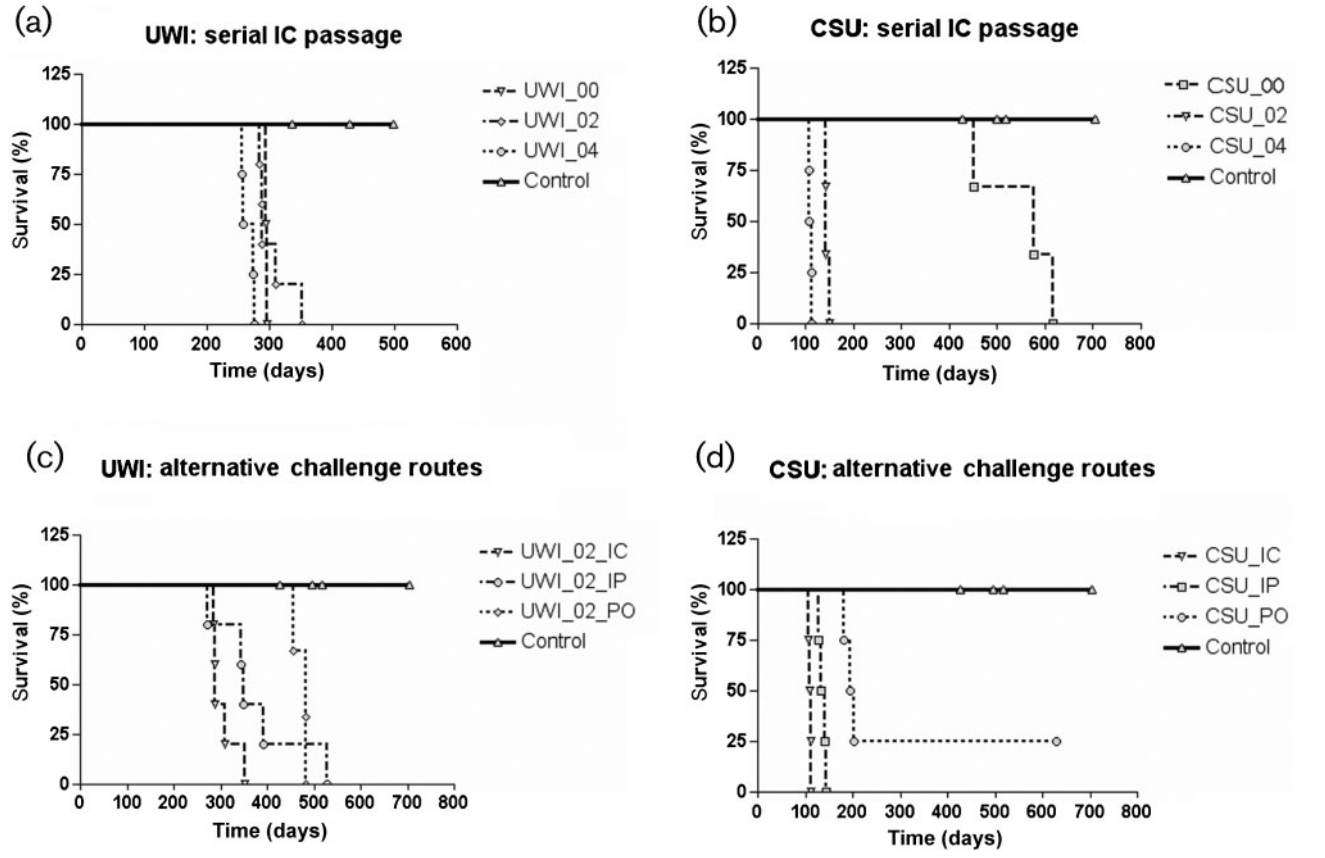

Fig. 1. Survival of ferrets inoculated by different routes with CWD-containing inoculum from two different sources. (a, b) Ferrets inoculated by the IC route with UWI isolate (a) and Colorado State University (CSU) isolate (b) showed stable or decreasing incubation periods upon serial passage respectively. (c, d) Ferrets inoculated with UWI isolate (c) and CSU isolate (d) by alternative routes were susceptible following intra-peritoneal (IP) inoculation and following exposure *per os* (PO). Year: _00 (2000), _02 (2002), _04 (2004).

with the CSU isolate progressed rapidly, spanning a 5–10 day (IC) or 10–20 day (IP/PO) period. In contrast, ferrets inoculated with the UWI isolate exhibited a more prolonged and variable disease course (3 weeks–3 months), leading to a larger spread in survival times (Fig. 1). This was most apparent for the IP route where a clinical plateau phase (>2 months) was sometimes observed.

Summary of transmission

CWD was transmitted to ferrets by IC inoculation, producing an AR of 100 % in all groups (Fig. 1). Greater variation was seen when cervid CWD was first passaged in ferrets than in subsequent ferret passages that were performed by IC inoculation. Ferret adaptation resulted in a tightly grouped pattern of onset consistent with a species barrier (Sigurdson *et al.*, 2008). Ferret-adapted CWD was efficiently transmitted by the IP and PO routes producing an overall AR of 94 %. For both isolates the survival times of ferrets infected via the IC, IP or PO routes were statistically different (UWI: $P=0.0072$, $P=0.0341$*; CSU: $P<0.0001$, $P=0.0116$*; *parametric and non-parametric statistics, respectively) (Fig. 1c, d).

Pathology and distribution of infectious prion protein (PrP^{CWD}) in the brain

The pathology of ferret CWD has been previously described (Bartz *et al.*, 1998; Sigurdson *et al.*, 2008). Using immunohistochemistry (IHC), we detected punctate and coarse granular aggregates of PrP^{CWD} in the brains of ferrets inoculated with the UWI isolate. We detected fine, granular, stippled and punctate aggregates of PrP^{CWD} that were variable in ferrets inoculated with the CSU isolate. In the brainstem and cerebellar regions, the CSU isolate appeared to show less PrP^{CWD} than the UWI isolate. In regions forward of the brainstem the comparison between the isolates was confounded by variable sensitivity of the IHC method, possibly attributable to variables in fixation time and protocols used to highlight PrP^{CWD} and diminish normal cellular prion protein (PrP^{C}) (formic acid, antigen

Table 2. Lymphoid tissues with PrP^{CWD} by isolate and route of inoculation

IHC, Immunohistochemistry; Western blotting, WB.

Lymphoid tissues tested for PrP^{CWD} by IHC and WB*	CSU isolate by route			UWI isolate by route		
	IC	IP	PO	IC	IP	PO
Spleen	–/–*	–/–	–/NT†	+/+‡	+/+	+/+
Mesenteric lymph node	–/NT	–/–	–/NT	+/+	+/+	+/+
Retropharyngeal lymph node	–/NT	–/–	–/NT	+/NT	+/+	+/+

*IHC/WB –/–, both tests negative.
†IHC/WB –/NT, negative/not tested by WB.
‡IHC/WB +/+, both tests positive.

retrieval, protease). Thus subjective differences in PrP^{CWD} deposition were not quantifiable by IHC.

Pathology and distribution of PrP^{CWD} in peripheral lymphoid tissues

There were no obvious gross lesions or histopathology detected in spleen or lymph nodes. For the UWI isolate, PrP^{CWD} immunostaining was detected consistently in the spleen, mesenteric and retropharyngeal lymph nodes irrespective of the route of challenge (Table 2, Fig. 2). PrP^{CWD} was concentrated in germinal centres of lymphoid follicles (Fig. 2). The proportion of follicles bearing PrP^{CWD} was usually less than 50 %. In contrast, PrP^{CWD} was not detected in lymphoid tissues of any ferrets inoculated with the CSU isolate (Table 2, Fig. 2a). This was confirmed using four PrP antibodies (BAR-224, L42, SAF-32 and 6H4). Amino-terminal directed, SAF-32 (Thielen *et al.*, 2001), and central-epitope directed, BAR-224 (Féraudet *et al.*, 2005) mAbs both detected PrP^{CWD} in lymphoid tissues of UWI-isolate-infected ferrets (Fig. 2b). The absence of PrP^{CWD} in lymphoid tissues of ferrets inoculated with the CSU isolate was verified using Western blot analysis and sodium phosphotungstic acid (NaPTA) precipitation. In contrast this method readily demonstrated PrP^{CWD} in lymphoid tissues of ferrets inoculated with the UWI isolate.

Molecular characterization of CSU and UWI isolates by Western blotting

Ferrets infected with either CWD isolate showed characteristic proteinase K (PK)-resistant bands spanning 17–29 kDa, as previously described (Bartz *et al.*, 1998; Sigurdson *et al.*, 2008). Despite marked differences in clinical disease and survival times, no difference was detected between the isolates in terms of electrophoretic migration or glycoform ratios. However, differences in PrP^{CWD} band intensities were observed between the two isolates, prompting investigation of their PK sensitivity.

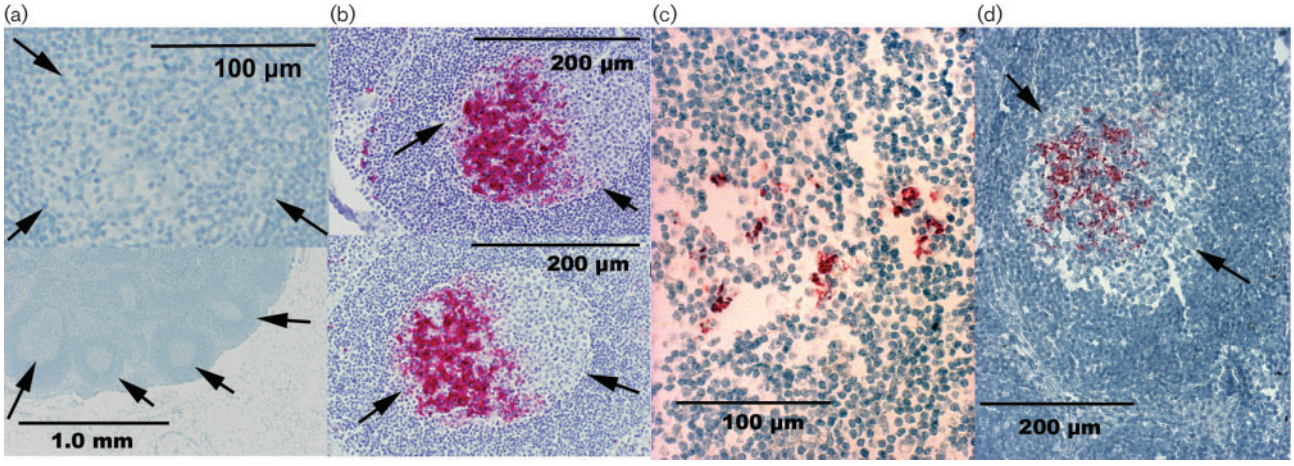

Fig. 2. Lymph nodes from ferrets inoculated with the CSU isolate (a) or UWI isolate (b, c, d) of CWD. (a) Complete lack of PrP^{CWD} (mAb BAR-224). (b) Serial sections of retropharyngeal lymph node, mAb BAR-224 (top) mAb SAF-32 (bottom). (c) Mesenteric lymph node (mAb L42). (d) Mesenteric lymph node (mAb BAR-224).

PK sensitivity

When an equivalent amount of PrP^{CWD}, estimated from densitometric analysis of undigested Western blotting signals, was digested with increasing concentrations of PK, PrP^{CWD} from the CSU isolate was more readily and completely degraded than PrP^{CWD} from the UWI isolate (Fig. 3). Three repetitions using minor variations in PK concentration and two repetitions using an alternative antibody (6H4) produced identical results (data not shown). The two CWD isolates differed significantly in their sensitivity to PK digestion.

Distribution of PrP^{C} in the brain

Molecular characterization showed regional differences in distribution of PrP^{C} in the ferret brain. The olfactory bulb had a unique PrP^{C} pattern and the cerebral cortex grey matter contained more PrP^{C} than the white matter (Fig. 4). Overall, the PrP^{C} signatures from different brain regions had more similarities than differences and were usually composed of a number of overlapping peaks in the range 20–37 kDa.

Distribution of PrP^{CWD} in the brain

Brains of end-stage ferrets inoculated by the IC, IP or PO routes with the CSU isolate had lower amounts of PrP^{CWD} in the brain than did ferrets challenged with the UWI isolate (Fig. 5). This was noticeable in the brainstem and cerebellar regions. When PrP^{CWD} signal was low in brains of infected animals (Fig. 5) undigested PrP^{C} immunoblots were examined to ensure PrP^{C} was present. For peripheral routes of exposure, the isolates were differentiated by accumulation in the olfactory bulb. All ferrets inoculated with the UWI isolate had substantial PrP^{CWD} signal whereas no ferrets inoculated with the CSU isolate had PrP^{CWD} detected in the olfactory bulb.

Densitometric capture of data from brain regions

To quantify observations in film-derived data for isolate-based patterns of PrP^{CWD} accumulation, analysis of scanned data was performed. Briefly, fluorescent output from one scanning pass was captured. A semi-quantitative, standardized and linear relationship between pixel density and the amount of PrP^{CWD} was determined. Routine inclusion of standards enabled comparison between

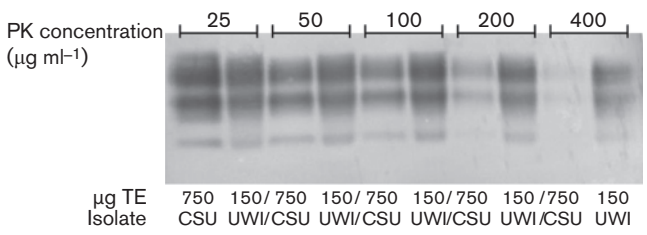

Fig. 3. Standard preparations, 750 or 150 μg tissue equivalents (TE), of brain from end-stage CWD-infected ferrets infected with either the UWI or the CSU isolate of CWD digested with increasing concentrations of PK.

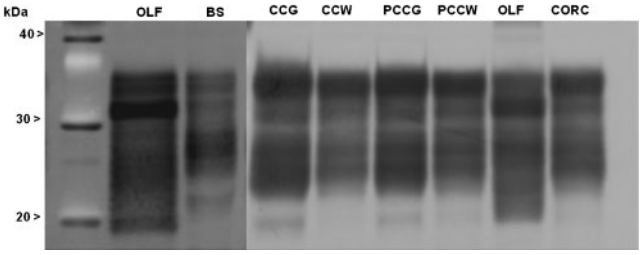

Fig. 4. The PrP^{C} profile from the olfactory bulb (OLF) was consistently different from that of other brain regions, for example brainstem (BS). Cerebral cortex/posterior cerebral cortex (CCW/PCCW) white matter and corpus callosum (CORC), contained lower concentrations of PrP^{C} than adjacent cerebral cortex/posterior cerebral cortex (CCG/PCCG) grey matter regions and OLF. A unique ~ 32 kDa PrP^{C} band was present in OLF.

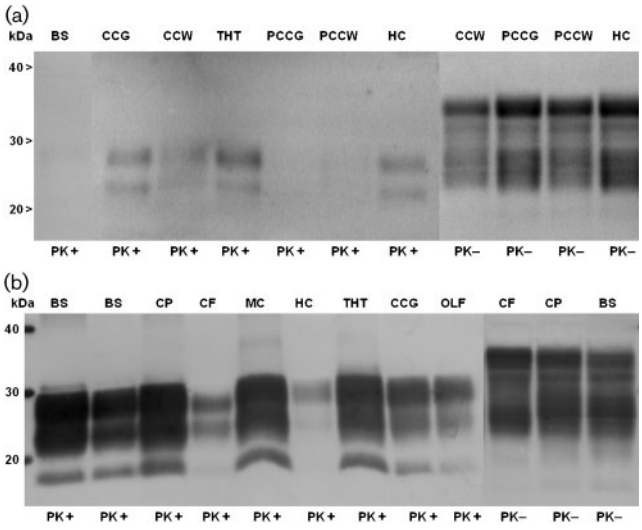

Fig. 5. Two representative experiments show PrP^{CWD} and PrP^{C} in a subset of brain regions. Ferrets were inoculated PO with either the CSU isolate (a) or the UWI isolate (b). A substantial difference in the amount and distribution of PrP^{CWD} was demonstrated when the two isolates were compared. For many brain regions of ferrets inoculated with the CSU isolate, PrP^{CWD} was at (BS, PCCG, PCCW) or below (CP, CF, MC, OLF) the limit for detection (undetectable signal not shown). BS, Brainstem; CCG, cerebral cortex grey matter; CCW, cerebral cortex white matter; CF, cerebellar folia; CP, cerebellar peduncle; HC, hippocampus; MC, midbrain colliculus; OLF, olfactory bulb; PCC, posterior cerebral cortex; PCCG, posterior cerebral cortex grey matter; PCCW, posterior cerebral cortex white matter; THT, thalamus. PK+, PK 50 μg ml^{-1} (PrP^{CWD}); PK–, no PK (PrP^{C}).

experiments. The absolute and relative accumulation of PrP^{CWD} in each brain region was determined. Patterns of accumulation were shown to be distinct for each isolate. For the UWI isolate a hind brain pattern of accumulation was noted, whereas for the CSU isolate relatively more PrP^{CWD} was detected in the forebrain. Examples (Fig. 6) wherein the distribution of PrP^{CWD} in ferrets inoculated with the CSU isolate differed notably from those inoculated with the UWI isolate is given for brainstem, cerebral cortex grey matter and olfactory bulb. Several other regions, including cerebellum, dorsal midbrain and cerebral cortex white matter also showed marked differences. Further information is given in the Supplementary Data section (available in JGV Online).

DISCUSSION

Two sources of cervid CWD, both from the original endemic area, exhibit distinct and reproducible clinical and biochemical features after adaptation to ferrets. Indicators of prion strain phenomena include differences in clinical presentation, survival period, distribution of lesions, glycoform profiles and in the resistance of the misfolded protein to proteolysis (Bessen & Marsh, 1992a, 1994; Bruce & Fraser, 1991; Bruce *et al.*, 1991; Everest *et al.*, 2006; Fraser & Dickinson, 1973). We encountered several of these indicators for 'strains' in CWD infection of ferrets, including differential PrP^{CWD} sensitivity to PK and differential distribution of PrP^{CWD} in the brain and lymphoid organs. The established susceptibility of ferrets to CWD (Bartz *et al.*, 1998; Sigurdson *et al.*, 2008) was extended to include IP and oral challenge. Shortening of the incubation period for the CSU isolate signalled a time course for infection that was quick for an outbred host and comparable with transgenic rodents (Browning *et al.*, 2004; Kong *et al.*, 2005; LaFauci *et al.*, 2006).

A major point of difference between isolates concerned accumulation of PrP^{CWD} in lymphoid tissues. Infection of ferrets with the CSU isolate never resulted in PrP^{CWD} in lymphoid tissue, including when NaPTA enrichment (Wadsworth *et al.*, 2001) and a panel of ferret PrP-recognizing antibodies was used. Replication in lymphoid tissue frequently precedes neural invasion and was presumed to be important in the pathogenesis of ferret CWD. Lymphoid accumulation of scrapie prion protein (PrP^{Sc}) or PrP^{CWD} is notable in scrapie and CWD, respectively (Andréoletti *et al.*, 2000; Sigurdson *et al.*, 1999), as opposed to bovine spongiform encephalopathy in which only minor accumulation occurs after experimental exposure (Terry *et al.*, 2003). Laboratory strains of scrapie demonstrate both relative and absolute differences in the distribution of PrP^{Sc} in the lymphoid tissues of a model host (Farquhar *et al.*, 1994). Furthermore, neuro-invasion without preliminary replication in the lymphoreticular system has been documented in hamsters inoculated with transmissible mink encephalopathy (TME) (Bartz *et al.*, 2005). The CSU isolate of CWD in ferrets appears to have features in common with this model of hamster TME in that a peripheral replication site was not identified in the current study.

Further differences between isolates of CWD were evident in the brains of inoculated ferrets and most apparent in the hind brain and olfactory regions. Many precedents exist for distinguishing prion strains by differences in the accumulation of misfolded protein and lesion characteristics in the brain (Bessen & Marsh, 1994; Bruce *et al.*, 1991; Fraser & Dickinson, 1973; Hecker *et al.*, 1992). Different sources of cervid CWD, inoculated into mice transgenic for elk PrP^{C}, showed differences in the distribution of PrP^{CWD} in the cerebellar and olfactory regions of the brain (LaFauci *et al.*, 2006). These two same regions of the brain showed differential accumulation of PrP^{CWD} between isolates in the current study.

The extent to which these unique CWD phenotypes in ferrets reflect more subtle phenomena in cervid populations

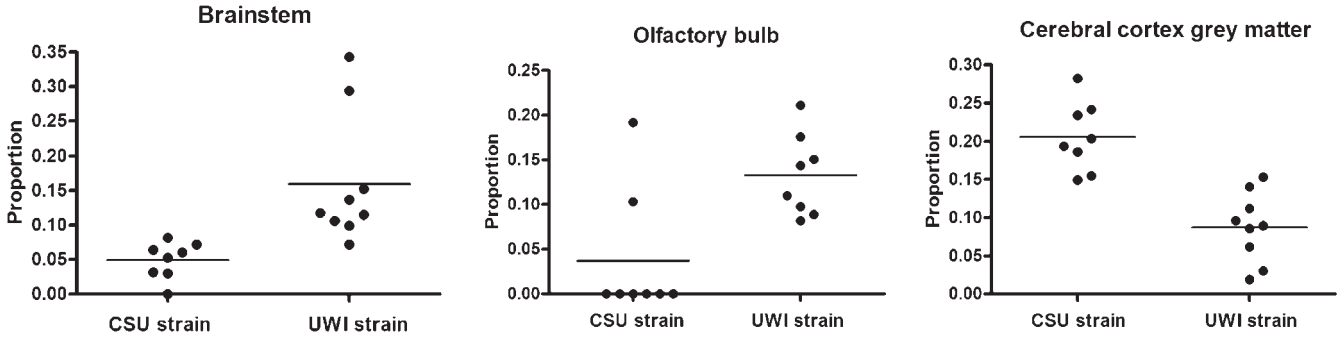

Fig. 6. Amount of PrP^{CWD} in a specified region of the brain region displayed as a proportion of the total amount of PrP^{CWD} in a standard subset of brain regions. Scatter plots with means (horizontal bar) illustrate statistically significant differences between the CSU and UWI isolates of ferret CWD for accumulation of PrP^{CWD} in the brain. A complete summary of differences is given in the Supplementary Data section.

remains to be determined. However, there is steadily increasing support for the existence of cervid disease subtypes and variants. Race *et al.* (2002) reported greater heterogeneity of glycoform patterns in mule deer PrP^{CWD} compared with elk PrP^{CWD} and speculated that this may indicate the existence of different or multiple cervid 'strains'. O'Rourke *et al.* (2007) described similar phenomena in elk with different *Prnp* genotypes. *Prnp* gene polymorphisms and pseudogenes exist and are linked to variation in susceptibility to CWD (Brayton *et al.*, 2004; Hamir *et al.*, 2006b; Huson & Happ, 2006; Johnson *et al.*, 2003, 2006; Kelly *et al.*, 2008; O'Rourke *et al.*, 1999, 2004). Mice transgenic for either leucine or methionine at codon 132 of the elk *Prnp* gene show absolute differences in susceptibility to CWD when challenged with different genotypes of CWD (Green *et al.*, 2008). Furthermore, cell-free conversion assays that have propagated mule deer CWD prions *in vitro* show that conversion efficiency is influenced by the amino acid sequence of the PrP^{C} template (Kurt *et al.*, 2009; Raymond *et al.*, 2007). The mechanism(s) by which amino acid sequence differences in PrP^{CWD} determine conversion kinetics is not well understood. What is evident is the potential for structural diversity of both PrP^{C} and PrP^{CWD} in the outbred cervid population. As further precedent, it was recently shown that distinct incubation period and neuropathological lesion profiles exist when cervid PrP^{CWD} from diverse sources are inoculated into transgenic mice (Angers *et al.*, 2010).

It was remarkable that ferrets inoculated by different routes showed essentially similar patterns of PrP^{CWD} accumulation in the brain. One possible explanation is that PrP^{CWD} from each isolate was interacting with different isoforms of PrP^{C}. In this way the PrP^{C} distribution would influence the distribution of PrP^{CWD} more strongly than the route of inoculation. Differences in native forms of the PrP^{C} molecule, its glycoform characteristics and distribution have been described (Beringue *et al.*, 2003), with *in vitro* models supporting the influence of PrP^{C} and glycosylation on conversion kinetics (Lawson *et al.*, 2005; Priola & Lawson, 2001). Because stereotactic inoculation of the brain was not used in this study, we consider peripheral, particularly natural, inoculation routes to be more informative regarding the differences between the isolates in terms of distribution of PrP^{CWD} in the brain.

What seems possible is that the unique CSU isolate arose from a different CWD variant or variants, pre-existing in the pooled mule-deer brain inoculum, and that passage of pooled cervid PrP^{CWD} in ferrets (Sigurdson *et al.*, 2008) selected for a dominant conformer. There was some evidence from Western blotting that this may have begun upon primary passage when cervid PrP^{CWD} proteins first encountered the species barrier. Presumably this process of selection of a dominant CWD conformer was irrelevant in the ferret-adapted UWI inoculum, because of its prior origin from a single cervid donor (Bartz *et al.*, 1998). If the cervid inoculum pool contained variants, then a single phenotype or kinetically 'fit' population of PrP^{CWD} molecules may have predominated. While *de novo* generation of prion strains has been described (Bartz *et al.*, 2000), co-inoculation trials in a well-characterized hamster TME model also provided evidence that prion conformer/strain interference is a mechanism influencing prion replication (Bartz *et al.*, 2007; Schutt & Bartz, 2008).

Distinct ferret-adapted CWD strains were identified in the present study and may have had an origin in the mule deer-derived inocula. Raymond *et al.* (2007) performed numerous passage experiments in hamster species using individual or pooled elk, mule deer and white-tailed deer inocula. From data obtained in Syrian golden hamsters, these authors suggested the existence of strains in cervid-derived inocula. Inocula from individual mule deer and elk gave distinctly divergent strains of hamster CWD based on incubation period and clinical signs. It would be interesting to determine whether ferret CWD, the CSU isolate in particular, retains pathogenicity for deer or cervid PrP-transgenic mice, with these studies now being relatively feasible (Browning *et al.*, 2004; Kong *et al.*, 2005; LaFauci *et al.*, 2006). There may be analogies with the hyper and drowsy strains of the TME agent (Bessen & Marsh, 1992a, b) wherein the drowsy strain, but not the hyper strain, retained its pathogenicity for mink.

The ferret is an outbred model for studies into the transmission and pathogenesis of CWD and has shown further utility as an alternative species. The ferret passage studies reported here parallel many investigations and observations that indicate the existence of TSE variants in ruminant host populations that are susceptible to prions (Benestad *et al.*, 2003; Buschmann *et al.*, 2004; Casalone *et al.*, 2004; Everest *et al.*, 2006). The results of these studies support the growing evidence for multiple strains of CWD prions.

METHODS

Animals. Disease-free domestic ferrets (*Mustela putorius furo*) were obtained as neutered weanlings (Marshall Farms, Wisconsin). Ferrets were housed in groups of two to five with *ad libitum* food and water. Dedicated utensils were maintained for each experimental cohort with handling protocols designed to enforce biosafety, biosecurity and separation. Excluding pilot studies, group size was determined by power analysis, to show incubation period differences of 1 month (http://stat.ubc.ca/~rollin/stats/ssize/n2.html). Animal ethics approval and guidelines were specific for the study (Colorado State University – Animal Care and Use Committee).

Inocula. The CSU inoculum originated from a mule-deer brain pool passaged once in ferrets (Sigurdson *et al.*, 2008). The pooled inoculum that was used to challenge the initial ferrets came from six captive mule deer that were naturally infected and were euthanized with clinical terminal CWD. The PK-resistant prion protein (PrP^{CWD}) was confirmed using IHC performed on brain sections at the level of the obex as well as tonsil. The distribution of PrP^{CWD} was consistent amongst the deer that were used to provide the pool and most prominent around the dorsal motor nucleus of the vagus nerve. Although the *Prnp* gene sequence of deer contributing to the inoculum pool would be advantageous, no fresh tissue from those deer remains.

The UWI inoculum, passaged twice in ferrets, was a gift from Dr Jason Bartz (Creighton Medical School) and originated from a single mule deer clinically affected with CWD (Bartz *et al.*, 1998). The geographical source of this individual mule deer and the six CWD-positive mule deer which constituted the CSU inoculum pool was the original endemic region for CWD.

Ferret inoculations. CWD-positive inocula, 10 and 25 % brain homogenates, were prepared in normal saline containing penicillin-streptomycin (100 U ml^{-1}) from the brains of ferrets with CWD. Ferrets were anaesthetized (atropine 0.05 mg kg^{-1}/telazol 20 mg kg^{-1}; Fort Dodge), and 300 or 1000 µl of 10 % suspension inoculated by the intra-cerebral (IC) or intra-peritoneal (IP) route, respectively. IC inoculation was into the left parietal cortex and used lidocaine (0.5 ml at the inoculation site; atropine and lidocaine were widely available as generic preparations for injectable use in veterinary medicine and research) and torbugesic (0.1 mg kg^{-1} intramuscularly; Fort Dodge) for analgesia. Exposure PO was by syringe feeding 1 ml of 25 % suspension on 3 consecutive days. Control ferrets were inoculated with brain suspensions prepared from a negative ferret.

Western blotting. To identify PrP^{CWD}, tissues were homogenized to 10 % (w/v) in PBS (pH 7.4) using a Fast-Prep (Thermo-Savant). Benzonase and $MgCl_2$ (Sigma) were added to a final concentration of 100 U ml^{-1} and 1.5 mM, respectively, and incubated (30–45 min, 37 °C) with agitation. Samples were agitated in an equal volume of 4 % (w/v) sarcosyl (Sigma) in PBS (30 min). Samples were incubated (1 h, 37 °C) with 50 µg proteinase K (PK; Invitrogen) ml^{-1}. Digestion was stopped with Pefabloc-SC (Fluka) at a 4 mM final concentration. Aliquots were boiled in sample buffer (Invitrogen), electrophoresed (12 % Bistris polyacrylamide gels/MOPS buffer system; NuPage Invitrogen) and transferred to PVDF membranes (Bio-Rad) using a Hoeffer tank and NuPage transfer buffer (Invitrogen). Blocking in 6 % (w/v) non-fat dried milk in TBS, 0.05 % (v/v) Tween 20 (Sigma) preceded incubation for 1 h with mAb Bar-224 (a gift from Dr Jacques Grassi, CEA/Saclay, France) at a final concentration of 0.066 µg ml^{-1} in blocking buffer. Secondary antibody, goat anti-mouse IgG FAb conjugated to HRP (Jackson Laboratories), was applied for 30 min (0.045 µg ml^{-1} in blocking solution). Blots were developed by chemiluminescence (ECL-plus; GE-HealthCare). Images were captured on film and with a STORM 860 scanner (Molecular Dynamics). Western blots were also developed with primary mAb SAF-32 (gift from Dr Grassi) and 6H4 (gift from Dr Bruno Oesch, Prionics AG, Zurich) at optimized dilutions between 1 : 1000 and 1 : 4000 (0.25–1.0 µg ml^{-1}).

NaPTA precipitation. When the concentration of PrP^{CWD} was low, NaPTA precipitation, as previously described (Wadsworth *et al.*, 2001), was beneficial. Briefly, following PK digestion and Pefabloc-SC, samples were centrifuged (1000 ***g***, 1–2 min) and supernatant transferred to a new tube. NaPTA (Sigma-Aldrich) was added to a final concentration of 0.3 % (w/v) and PrP^{CWD} pelleted by centrifugation (18 000 ***g***, 30 min). The pellet was resuspended in PBS containing 0.1 % (w/v) sarcosyl. Concentrated samples were electrophoresed and PrP^{CWD} detected as above.

PK sensitivity. Brain homogenates were prepared from ferrets with advanced CWD following IC inoculation. Tissue aliquots from standard regions were adjusted to 5 % tissue (w/v) in 2 % (w/v) sarcosyl in PBS and digested with a range of PK concentrations (6.25–400 µg ml^{-1}) for 1 h. Western blots were developed with primary mAbs BAR-224, SAF-32 and 6H4 at optimized dilutions between 1 : 1000 and 1 : 4000 (0.25–1.0 µg ml^{-1}).

IHC. Tissues were both fixed by perfusion and post-fixed with 4 % paraformaldehyde–lysine–periodate (McLean & Nakane, 1974). Tissues were treated with 88 % formic acid (Sigma) for 1 h prior to tissue processing. For antigen retrieval, tissues were immersed in 88 % formic acid for 5–15 min, autoclaved at 121 °C for 10–15 min in target antigen retrieval solution (Dako), and/or treated with cell conditioning solution, CC1 (Ventana medical systems; Roche). Several anti-PrP antibodies detected PrP^{CWD} immunostaining in ferrets. The most successful were BAR-224, SAF-32, 6H4 and L42 (R-Biopharm AG). BAR-224 and SA-32 were routinely used at optimized concentrations (0.25–10 µg ml^{-1}). A 'Discovery' immunostainer and proprietary (RedMap) kit was used (Roche). User-defined steps included protease III digestion (2 min), biotin blocking (4 min) and mAb BAR-224 (0.25–4.0 µg ml^{-1}). Optimization was tissue specific. Lymphoid tissue from ferrets was tested against all antibodies showing reactivity to ferret PrP^{CWD}.

Controls. Uninfected tissue sections with the antibody applied were included in each run. Omission and substitution of the primary reagent with an irrelevant antibody was used to confirm the specificity of IHC for each tissue type.

Brain region analysis. Longitudinal hemi-sections of brains from 17 CWD-infected ferrets were sectioned transversely at defined anatomical landmarks. Up to 12 specific brain regions were collected for Western blot analysis. Cerebral cortex samples were taken from the same region of the parietal cortex in each ferret and grey and white matter separated from that sample. A consistent sampling method was used for each ferret and each brain region. Samples were adjusted to 10 % (w/v) and 800 µg tissue equivalents analysed by Western blotting as above. Digitized image data (Storm scanner and Image Quant software version 5.1; Molecular Dynamics) was used to compare PrP^{CWD} in each brain region. Comparisons were based on densitometric volume analysis using the diglycosylated band. Analysis of PrP^{C} utilized a 5 % (w/v) tissue suspension. PK digestion was omitted and the brain region comparison based on 125 µg tissue equivalents.

Statistical analysis. Analyses of incubation period data used a *t*-statistic, Mann–Whitney and log rank tests. The generation of Kaplan–Myer survival curves was performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, www.graphpad.com). For analysis of densitometry data, ImageQuant version 5.1 was used with standardized options for background correction and the results exported to Microsoft Excel. Collation and matching of datasets was done using ImageQuant and Excel with statistical analyses (*t*-tests and ANOVA) performed using GraphPad. Further assistance was provided by Dr Philip Chapman, Department of Statistics, Colorado State University.

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